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Particle activity and in vivo pulmonary response to freshly milled and aged alpha-quartz

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This study examined the possibility of freshly fractured α-quartz being more toxic and inflammatory in vivo than aged quartz of the same composition and particle size. Fresh quartz was generated by a jet mill, and used immediately, while aged dust was stored for two months before use. Both the production of hydrogen peroxide and hydroxyl radicals and the analysis of surface radicals verified the enhanced surface activity of fresh quartz. Male Fischer 344 rats were exposed to fresh or aged α-quartz by inhalation (20 mg · m^{-3}, 5 h per day, 5 d per week, for 2 weeks) and their pulmonary responses were determined 1—3 d postexposure. Exposure to aged quartz resulted in an increase in cytotoxic and inflammatory parameters. In comparison, the inhalation of freshly cleaved quartz resulted in dramatically greater increases in all of the pulmonary responses. This finding suggests that exposure to freshly machined quartz may result in a greater risk of pulmonary disease.

Key terms aged, freshly fractured silica, inhalation, pulmonary inflammation, silicosis, surface activity.

Exposure to quartz dust is an occupational hazard affecting many workers, including miners, sandblasters, and glass workers. Occupational and environmental monitoring for quartz, as well as the exposure limits, are all concerned with the concentration of the dust. However, quartz that has been recently fractured has been shown to have increased surface activity. Hoenig (1) has shown that grinding quartz (and other materials) causes an exo-electron current. Göthe et al (2) found that disintegrated quartz dust causes an increase in the lung weight and collagen content of rats in comparison with rats exposed to "standard quartz" of comparable particle size. Conversely, Bar-Ziv & Goldberg (3) suggested that the lack of fibrosis in the lungs of Bedouins in which siliceous dust was present was due to the age of the inhaled particulate. More recently, fracturing has been shown to enhance the ability to cause membrane damage, the peroxidation of membrane lipids, and activation of alveolar macrophages in vitro (4—7). These results and previous suggestions imply that workers exposed to freshly fractured silica may be at increased risk of developing pulmonary injury.

To investigate these questions, an inhalation exposure study involving 900 rats has been planned. The study requires the exposure of 300 rats, each to equivalent concentrations of freshly fractured and aged respirable quartz dust, with an additional 300 rats as controls. In this context, aged silica is milled quartz of the same particle size that has aged in air for at least two months. At the end of 2, 4, and 26 weeks, rats from each exposure group will be sacrificed and a series of biochemical and pathological tests performed to determine alveolar damage, cellular inflammation, and alveolar macrophage activation.

To evaluate the entire experimental protocol adequately, a two-week pilot study was performed. All of the procedures planned for the full exposure were evaluated, including the quartz aerosol generation systems, sampling procedures, aerosol analyses (concentration, particle size), dust activity analyses, animal care and handling, and biochemical and pathological analyses. This report summarizes the experimental protocol and presents the results of the two-week pilot study.

Methods

Exposure. In brief, aerosols of quartz dust (20 mg · m^{-3}) were generated in two chambers, each housing 20 male Fischer 344 rats. Quartz sand was air-jet milled, passed through a cyclone, and immediately directed into the "fresh" chamber. The aerosol of previously milled quartz dust that had aged two months was prepared by dropping the dust onto a revolving plate and resuspending the dust through aspiration. This aerosol was

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introduced into the "aged" chamber after passage through a cyclone. The control rats were housed in a chamber into which only filtered air passed. The exposures were performed 5 h each workday for two weeks, and pulmonary responses were determined 1—3 d postexposure. The aerosols were monitored by gravimetry and particle counters for concentration by scanning electron microscopy for particle size and by electron spin resonance (ESR) and the chemical methods for activity levels (hydrogen peroxide, hydroxyl and surface radicals).

The quartz used in this study was Iota standard quartz sand (Unimin Corporation, New Canaan, Connecticut, United States) with a mass median diameter of 193 μm. An optical microscopic examination revealed that the material was at least 99% quartz.

Since metals can greatly influence surface activity and radical production, proton-induced X-ray emission spectroscopy was performed for 72 trace elements, both before and after the milling. The elements that were consistently detected in all of the samples are shown in table 1. These contaminants are a result of the aerosol generation systems. The carbon is assumed to come from the abrasion of the polyurethane liner of the air-jet mill. The iron, chromium, nickel, and manganese are from the stainless steel screws feeding the generation systems. An examination of filter samples collected from the exposure atmosphere by scanning electron microscopy revealed discrete particles of stainless steel agglomerated with several quartz particles. Steps have been taken to reduce the amount of metal contamination for the larger study.

The quartz aerosol generation systems worked well. The time-weighted average concentrations were 22.4 (range 19.9—36.2) $mg \cdot m^{-3}$ for the fresh chamber and 19.3 (range 17.1—20.7) $mg \cdot m^{-3}$ for the aged chamber. The temperature, humidity, and ammonia concentration remained in control and were not significantly different in the three chambers. The count median of the circular-area equivalent diameter for the fresh dust was 0.46 μm with a geometric standard deviation of 2.1; for the aged dust the corresponding values were 0.53 μm and 2.2, respectively.

Measurement of particle activity. The peroxidase-catalyzed oxidation of 4-hydroxyphenylacetic acid (PHPA) by hydrogen peroxide yields a strongly fluorescent dimer of PHPA (8). In our application, the quartz-laden filter was placed into a buffer solution (pH 7) and sonicated for 1 min to remove the dust. The suspension was agitated at 25°C for 30 min to allow efficient transfer of hydrogen peroxide into the solution. The supernatant was combined with PHPA and peroxidase and adjusted to pH 10 after 30 min (to allow) for reaction. The analysis was carried out with a flow injection with a fluorescence detector (excitation 320 nm, emission 420 nm).

Table 1. Elements consistently detected in the quartz dust analyses.

Elements	Bulk[a] ($\mu g \cdot g^{-1}$)	Milled[b] ($\mu g \cdot g^{-1}$)
Carbon[c]	54	1654[d]
Chromium	<1.2	58
Iron	7.0	222
Manganese	0.93	6.6
Nickel	0.56	25

[a] Based on an analysis of three samples.
[b] Based on an analysis of six samples.
[c] Carbon was analyzed with an induction furnace method.
[d] Based on an analysis of seven samples.

For determining hydroxyl radical, the dust was scraped from the filter into the 2-deoxyguanosine solution, agitated for 1 h to allow generation of the hydroxyl radical and the production of 8-hydroxy-2-deoxyguanosine, and then centrifuged. The supernatant was then analyzed by high-performance liquid chromatography with electrochemical detection at +0.3 V (9, 10).

The surface reactivity of freshly milled and aged quartz was determined with ESR spectroscopy. On three days, the quartz dust from triplicate filter samples from the exposure chambers was transferred to 5-mm quartz nuclear magnetic resonance tubes and analyzed for free radicals with a Varian E-109 ESR spectrometer at X-band (~9.52 GHz). All of the measurements were made at a receiver gain of $1 \cdot 10^4$, a microwave power of 50 mW, a time constant of 1 s, modulation amplitude of 2 gauss, and a scan time of 120 s with a field amplitude of 3380 (SD 200) gauss. Three scans were integrated for all of the samples, and the scaling and analysis of the spectra were made with an electron paramagnetic resonance DAP 2.0 program. The silicon-based surface radicals of freshly fractured and aged quartz were typically centered around 2.0015 g, and the surface activity was determined from the peak intensities relative to a standard sample of diphenylpicrylhydrazyl (4, 11).

Pulmonary responses. Pulmonary responses were determined 1—3 d postexposure. Briefly, rats were anesthetized and then killed, and bronchoalveolar lavage was performed. The lavage samples were separated into acellular, for the determination of lavage protein, phospholipid, albumin, and N-acetyl-β-D-glucosaminidase (NAG), and cellular, for the determination of cell differentials and macrophage activity fractions. Another set of rats was also killed, and lung slices were analyzed for lipid peroxidation.

The protein content of the acellular lavage fluid was measured by the method of Lowry et al (12). The total phospholipid concentration was determined as the phosphorus present in the lipid extract (13). The phospholipid content was calculated by multiplying the lipid phosphorus values by 25 (14). Albumin was measured with Sigma Diagnostic reagents and procedures. NAG was determined by measuring the release of 3-cresolsulfonphthalcin from the substrate 3-cresolsulfonphthalcin-N-acetyl-β-D-glucosaminide at 580 nm according to the method of Yakada et al (15).

Bronchoalveolar lavage cells were collected, washed, and resuspended in HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM of HEPES, 5.5 mM of glucose, and 1 mM of $CaCl_2$; pH 7.4). Cell counts and differentials were obtained with an electronic cell counter equipped with a cell sizing attachment (16). The chemiluminescence generated from the pulmonary phagocytes was measured with a Berthold LB953 luminometer at 37°C in the presence of 8 μg% luminol and 2 $mg \cdot ml^{-1}$ zymosan.

The lipid peroxidation potential of the lungs from the animals exposed to filtered clean air, aged quartz, or freshly fractured quartz was monitored by measuring the malondialdehyde generated during the incubation of lung slices for 1 h in a buffered medium without any other stimulation. Frozen lung tissue slices (300—450 mg) were incubated in phosphate-buffered medium at pH 7.4 for 1 h at 37°C in a shaking water bath. The reaction was terminated by the addition of 0.3 ml of 5N hydrochloric acid and 0.625 ml of 40% trichloroacetic acid. After being mixed, the reaction was treated with 0.625 ml of thiobarbuturic acid, mixed, and heated in a water bath at 95°C for 20 min. The substances reactive

to thiobarbuturic acid developed a color which was measured at 540 nm after cooling according to the method of Hunter et al (17). Malondialdehyde production was calculated from a standard graph made with the same reagents and known concentrations of malondialdehyde. Control experiments were carried out without lung tissues, with inactivated lung tissue, and in the presence of an antioxidant, butyl hydroxytoluene, to inhibit lipid peroxidation.

Results

As we expected, the activities of the quartz in the two aerosols were significantly different (figure 1). The hydrogen peroxide levels (95% confidence intervals) were 0.256 ± 0.018 nmol · mg^{-1} for the freshly fractured quartz and 0.114 ± 0.026 nmol · mg^{-1} for the aged samples, a 125% difference. The hydroxyl radical level produced in 1 h by the fresh quartz was 27.13 ± 4.07 pmol · $sample^{-1}$, while the aged quartz produced 19.04 ± 3.43 pmol · $sample^{-1}$, a 43% difference. The ESR measurements showed that fresh quartz produced $1.46 \pm 0.17 \times 10^{13}$ spins · mg^{-1} in comparison with $0.96 \pm 0.11 \times 10^{13}$ spins · mg^{-1} for the aged quartz, a 52% difference.

The inhalation of aged α-quartz (20 mg · m^{-3}) caused significant cytotoxicity and pulmonary inflammation (table 2). Damage at the alveolar blood-air barrier was indicated by increased lavage levels of red blood cells, albumin, and protein. Damage at the cell and membrane level was shown as an elevation of the lavage levels of NAG and by an increase in the lipid peroxidation of lung tissue. A lipidotic response to aged quartz was evidenced by elevated levels of phospholipid in lavage fluid, while inflammation was demonstrated as an increase in lavage leukocytes and an enhanced generation of zymosan-stimulated chemiluminescence from alveolar macrophages. In comparison with aged dust exposure, the inhalation of freshly milled quartz resulted in significantly greater reactions for all of these cytotoxic and inflammatory parameters.

Discussion

Recent reports indicate that crushing silica particles results in the generation of silicon-oxygen radicals on the cleavage planes (4, 18). In addition, freshly ground silica can react in aqueous media to generate hydroxyl radicals (19). The present investigation describes an air-jet mill system capable of generating sufficient quantities of freshly cleaved α-quartz for inhalation studies. The enhanced activity of this freshly milled dust, compared with milled dust which was stored for two months, was verified by the increased production of hydrogen peroxide and hydroxyl radicals and by an enhanced ESR signal indicative of silicon-based surface radicals.

In vitro studies indicate that freshly crushed silica is more cytotoxic and is a more potent activator of oxidant release from alveolar macrophages than ground silica which has been aged to allow the decay of these surface radicals (4, 5). Therefore, the hypothesis has been advanced that freshly machined particles would be more fibrogenic than aged dust. Results of the present inhalation study confirm in vivo the enhanced activity of the freshly fractured silica dust relative to that of aged dust. The freshly machined quartz exhibited greater cytotoxicity than aged particulates, which in turn produced a significantly greater response than that found in the control experiment. This cytotoxicity was demonstrated by elevated red blood cell counts, albumin, and protein in lavage samples, all of which indicated damage at the blood-airspace barrier, and by increased enzyme levels and lipid peroxidation, indicating damage at the cell and membrane level. Likewise freshly milled silica was more inflammatory than aged quartz as evidenced by the greater recruitment of leukocytes into the airspaces and the enhanced potentiation of alveolar macrophage oxidant production.

As shown in figure 1, the activity of the aged silica used on days 9 and 10 of this pilot study was much higher than the

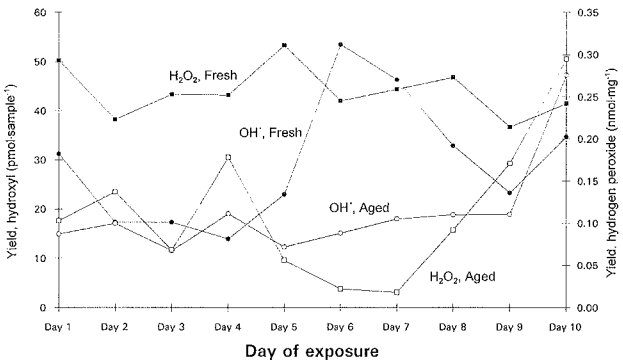

Figure 1. Yields of hydrogen peroxide (H_2O_2) and hydroxyl radical (OH·). Each point is the average of two to five samples taken during each day.

Table 2. Pulmonary responses to the inhalation of fresh versus aged α-quartz. Values are means and standard deviations of four determinations; lavage red blood cells (RBC) and leukocytes in 10^6 cells per rat; albumin in micrograms per microliter of acellular lavage fluid; protein and phospholipid from acellular lavage samples in milligrams per gram of lung; N-acetyl-ß-D-glucosaminidase (NAG) in units per liter of acellular lavage fluid; lipid peroxidation in micromoles of malondialdehyde per gram of lung tissue; and zymosan-stimulated chemiluminescence (Zymo-stim CL) in counts per minute/0.75×10^6 macrophages per 10 min.

Parameter	Air		Aged silica		Machined silica	
	Mean	SD	Mean	SD	Mean	SD
RBC	0.10	0.03	1.48	0.14[a]	5.83	0.74[b]
Albumin	0.3	0.3	0.34	0.09	0.62	0.05[b]
Protein	2.76	0.33	3.93	0.15[a]	4.97	0.03[b]
NAG	35.0	0.0	53.42	10.91[a]	126.52	15.78[b]
Lipid peroxidation	1.01	0.16	2.27	0.37[a]	3.02	0.29[b]
Phospholipid	1.71	0.03	3.66	0.19[a]	5.12	0.15[b]
Leukocytes	0.10	0.01	4.75	0.66[a]	10.70	1.33[b]
Zymo-stim CL	29.34	2.41	277.82	13.08[a]	767.54	79.85[b]

[a] Significantly greater than control ($P < 0.05$).
[b] Significantly greater than aged silica ($P < 0.05$)

dust used the previous eight exposure days (for a variety of technical errors). This increase in activity acts to minimize the differences in the pulmonary responses of the fresh versus aged quartz groups. However, substantial cytotoxic and inflammatory differences were evident that lend support to the hypothesis that freshly cleaved silica may be more pathogenic than aged quartz. These technical errors have been corrected in preparation for the larger study.

In conclusion workers such as sandblasters, rock drillers, and silica flour millers exhibit a significant incidence of silicosis. A factor common to these occupations is exposure to freshly broken or fractured quartz particles. Data from both in vitro and in vivo studies indicate that freshly cleaved silica is more cytotoxic and inflammatory than aged quartz of the same particle size at similar exposure doses. Data suggest that increased disease risk may be related to the unique activity of the fresh cleavage planes of the broken α-quartz crystal.

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